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MALARIA AND POLYPEPTIDES OF *PLASMODIUM FALCIPARUM* AT THE INFECTED ERYTHROCYTE SURFACE

Mia Palmkvist

Karolinska Institutet

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MALARIA AND POLYPEPTIDES OF *PLASMODIUM FALCIPARUM* AT THE INFECTED ERYTHROCYTE SURFACE
THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

Mia Palmkvist

Principal Supervisor:
Prof. Mats Wahlgren
Karolinska Institutet
Department of Microbiology, Tumor and Cell Biology (MTC)

Co-supervisor(s):
Dr. Johan Ankarklev
Karolinska Institutet
Department of Microbiology, Tumor and Cell Biology (MTC)

Prof. Björn Andersson
Karolinska Institutet
Department of Cell and Molecular Biology (CMB)

Opponent:
Prof. Thor G Theander
University of Copenhagen
Department of Immunology and Microbiology (ISIM)

Examination Board:
Dr. Jose Pedro Gil
Karolinska Institutet
Department of Physiology and Pharmacology (FYFA)

Prof. Hannah Akuffo
Karolinska Institutet
Department of Microbiology, Tumor and Cell Biology (MTC)

Dr. Lucia Cavelier
Uppsala University
Department of Immunology, Genetics and Pathology

Till Daniel & Vilda

ABSTRACT

Malaria is one of the top three most lethal infectious diseases in the world after tuberculosis and HIV. The disease is caused by intracellular parasites of the genus *Plasmodium spp.* To date, five *Plasmodium spp* have been linked to human infection, where *Plasmodium falciparum* is known to cause the most severe forms of the disease, killing upwards of 600 000 people per year. The parasite has a complex life cycle that involves two hosts, the Anopheles mosquito and the human. Humans become infected when bitten by an infected mosquito. In the human host the parasite divides asexually inside the red blood cells, each infected erythrocyte gives rise to 16-32 merozoites that egress and reinvade new red blood cells. A small subset of the parasites also sexually commit and become female or male gametocytes that are crucial for the transmission back to the mosquito. The intra erythrocytic cycle is responsible for causing the symptoms in the human host. During development in the red blood cell the parasite transports and expresses polypeptides on the cell surface making the membrane of the infected erythrocyte "sticky". These sticky proteins mediate adhesion to uninfected red blood cells forming, so called "rosettes". Moreover, these proteins can also bind to endothelial cells in the microvasculature, a phenomenon known as "sequestration", which leads to severe obstruction of the blood flow in the host. Rosetting and sequestration are major pathological features of severe malaria. This thesis focuses on the polypeptides involved in parasite sequestration and rosetting. We identified the RIFIN protein family as a major mediating factor in rosetting and describe how the binding properties are dependent on the ABO blood group antigens of the human host. In addition we utilized single cell RNA-Seq to study differential gene expression, of important gene families that are linked to sexual commitment, sequestration and rosetting, in malaria-infected red blood cells at single cell resolution.

These discoveries regarding the RIFINs are key findings in the development of novel drugs/vaccines against severe malaria disease.

LIST OF SCIENTIFIC PAPERS

This thesis is based on the following papers, which will be referred in the text by their Roman numbers.

I. Goel S*, **Mia Palmkvist***, Moll K*, Joannin N, Lara P, Akhouri RR, Moradi N, Öjemalm K, Westman M, Angeletti D, Kjellin H, Lehtiö J, Blixt O, Ideström L, Gahmberg CG, Storry JR, Hult AK, Olsson ML, von Heijne G, Nilsson I, Wahlgren M.
RIFINs are adhesins implicated in severe Plasmodium falciparum malaria.
Nature Medicine. 2015 Apr;21(4):314-7

II. Moll K, **Mia Palmkvist**, Ch'ng J, Kiwuwa MS, Wahlgren M.
Evasion of Immunity to Plasmodium falciparum: Rosettes of blood group A impair recognition of PfEMP1
PLoS One. 2016 Feb 12;11(2):e0149765

III. Ngara M*, **Mia Palmkvist*,** Sagasser S*, Ankarklev J, Björklund K. Å, Wahlgren M, Sandberg R.
Transcriptional heterogeneity of Plasmodium falciparum at cellular level
Manuscript

* Equal contribution

CONTENTS

LIST OF ABBREVIATIONS

Ab	Antibody
Bg	Blood group
CM	Cerebral malaria
CSA	Chondroitin sulphate A
FACS	Flow activated cell sorting
HS	Heparan sulphate
Ig	Immunoglobulin
mAb	Monoclonal antibody
MC	Maurer´s cleft
NTS	N-terminal segment
PEXEL	*Plasmodium* export element
PfEMP1	*Plasmodium falciparum* erythrocyte membrane protein 1
iRBC	Infected red blood cell
PTEX	*Plasmodium* translocon of exported proteins
PVM	Parasitophorous vacuolar membrane
RBC	Red blood cell
Rif	Gene encoding RIFIN protein
RIFIN	Repetitive interspersed family
Sp, Spp	Species
VSA	Variable surface antigen
WHO	World Health Organization

1 INTRODUCTION

1.1 THE HISTORY OF MALARIA

Malaria now known as a tropical disease is an ancient disease that has affected humankind for 500,000 years. It has been spread throughout the world killing billions of people and has had enormous impact on human colonization. The earliest written records of a disease describes symptoms and signs of malaria is dated back to approximately 2700 BCE in an ancient Chinese Canon of Medicine known as the Nei Ching. In 1570 BC malaria is also described in the ancient Egyptian medical text, the Ebers Papyrus. Ancient medical writings from India and Assyria also contain descriptions of malaria. In 400 BC Hippocrates describes in his book Epidemics periodical fevers and splenomegaly, which are typical malaria symptoms. He describes the seasonality of malaria and how mostly habitants living within close proximities close of swamps are affected and suggests that the illness is due to the miasma, foul air rising from the swamps. In the following 2000 years this was the belief and malaria got its name from Italian ”mala aria” which literally translates to bad air. It was first in 1676 when Antoni van Leeuwenhoek discovered that bacteria can cause infectious disease followed by the discoveries by Pasteur and Koch in 1880 that the search for a microorganism that causes malaria started. In 1897, an army officer working in Algeria named Charles Louis Alphonse Laveran was the first to find parasites in the blood of patients suffering from malaria and linking them to the disease (for which he won the Nobel Prize in 1907). This was followed by the discovery of mosquitos as the vector for avian malaria by the British army surgeon Ronald Ross in 1897 in India and subsequently for human malaria in Italy by Italian malarialogists Giovanni Battista Grassi, Amico Bignami, Giuseppe Bastianelli, Angelo Celli, Camillo Golgi and Ettore Marchiafava between 1898 and 1900. Grassi then completed the asexual life cycle of the malaria parasite in humans and the sexual life cycle in the Anopheles mosquito. A more detailed picture of the life cycle took 70 more years to discover [1, 2] [3]. Today the genomes of the malaria mosquito *Anopheles gambiae,* as well as the human infecting parasites, *Plasmodium falciparum* has been sequenced (2002) and our knowledge of both the vector and the parasite have greatly improved.

1.2 THE PRESENT DISEASE BURDEN

Malaria caused by *P.falciparum* is still a leading cause of human death. The WHO has estimated that 3.2 billion people in 97 countries are at risk of being infected with malaria. In 2013 approximately 198 million cases of malaria occurred globally and the disease led to 584 000 deaths. 90% of the deaths occurred in Africa and children under the age of 5 accounted for 78% of all deaths. (WHO annual report 2015).

The burden of malaria is heaviest in Africa south of the Sahara where the disease is most severe and affects the poorest communities that have very limited access to services of prevention, diagnostics and treatment.

1.3 THE MALARIA PARASITES

Malaria is a mosquito borne disease caused by unicellular eukaryotic protozoan parasites of the *Plasmodium spp.* The *Plasmodium spp* belongs to the Apicomplexan phylum that includes a range of other parasites such as; *Toxoplasma gondii, Cryptosporidium spp, Babesia spp,* which are capable of infecting humans and other warm-blooded animals. The phylum was named after the specific apical complex of the cells that contains organelles such as the micronemes and rhoptries that are used by the parasites use to invade their respective host cells. While there are more than 250 *Plasmodium spp* that can infect a wide range of different hosts such as birds, reptiles, rodents and monkeys. Five species have been identified to have the capacity of infecting humans these are *P.falciparum, P.vivax, P.ovale, P. malariae* and *P.knowlesi*. The majority of severe and lethal cases of malaria is caused by *P.falciparum* and is prevalent in sub-Saharan Africa but also found in tropical areas worldwide. P.vivax has a wider distribution due to its ability to develop in the Anopheles mosquito vector at lower temperatures and higher altitude. It is most common in Asia, Latin America and some parts of Africa and generally causes a milder disease. *P.ovale,* mostly found in Africa and *P.malariae,* which is found worldwide, are the two least frequent malaria species. *P.knowlesi* the fifth malaria species has the macaques as its natural host and was only recently recognized to infect humans. *P.knowlesi* is found in Malaysian Borneo [4] [5] [6] [7]

The scope of this thesis is the parasite P.falciparum and therefore the following chapters will focus mainly on this species.

1.3.1 The life cycle of Plasmodium spp

Malaria parasites are obligate intracellular organisms and the life cycle of *P.falciparum* is complex. It involves two hosts and several developmental stages. The parasite undergoes

sexual replication in the Anopheles mosquito (definite host) and asexual replication in humans (intermediate hosts).

The sporozoite is the stage that infects humans. Sporozoites develop within oocysts in the midgut of the blood feeding female Anopheles mosquito vector. Subsequently, the sporozoites are released from mature oocysts and travels to the mosquito salivary glands where they invade gland cells, go through the cytoplasm of the cells and enter the excretory cavity. Here they reside viable for weeks [8].

Humans become infected when the mosquito attempts to find a blood vessel for feeding, sporozoites are then released into the skin [9] [10] [11]. The injected sporozoites utilize gliding motility to move within the dermis [8] [12]. Due to their ability to enter and travers cell barriers some parasites reach the blood stream and are transported to the liver where they exit the blood vessel and migrate through several hepatocytes before infecting a final one [13] [12]. Several surface proteins play an important role for infection, such as the CSP (Circumsporozoite protein) [14] TRAP (thrombospondin-related adhesion protein) [15] [16] [17] and AMA1 (apical membrane protein 1) [18]. Invasion of the hepatocyte occurs with a formation of a vacuole inside the cell where the parasites can multiply mitotically and differentiate into thousands of merozoites [19] [13]. The merozoites are then packaged into merosome vesicles, each containing 100-200 merozoites, that are released after 5-15 days from the hepatocyte by budding and enter the blood circulation [20].

This stage that occurs in the human circulatory system is called the erythrocytic stage of the parasite life cycle. In the bloodstream the free merozoites rapidly invade red blood cells and start to multiply asexually inside a process termed erythrocytic schizogony. The parasites develop from a ring form into trophozoite and finally to a mature schizont that bursts the erythrocyte membrane, releasing 16-32 new merozoites which invade new un-infected red blood cells in a continuous cycle. Depending on the *Plasmodium spp* the cycle from ring stage to mature schizont takes between 24-72h, approximately 48h for *P.falciparum.* The erythrocytic stage is responsible for the clinical manifestations of malaria including fever, chills, fever peaks typically occur every 24, 48 or 74 h depending on the species and is caused by synchronous bursts of mature schizonts, which releases both parasites and erythrocytic material into the blood stream [21] [22] [23] [24].

A small fraction of the asexually replicating parasites develops into female and male gametocytes, the sexual forms of the parasites [25] [26] [27] [28]. The gametocytes develop in five distinct stages, while the inter -mediate stages are sequestered possibly in the bone marrow [29], the late stage gametocytes return to the bloodstream to make themselves

available to a feeding mosquito. In the midgut of the mosquito the gametocytes develop into female and male gametes, which fuse and form diploid zygotes that develop into ookinetes. The ookinete invades the midgut wall where it forms into an oocyst that further matures and divides in a process termed sporogony, generating thousands of sporozoites. When the oocyst ruptures the sporozoites are released and migrate to the salivary gland of the mosquito ready to be injected into the human host during the next blood meal, thus completing the *Plasmodium* sexual life cycle [30].

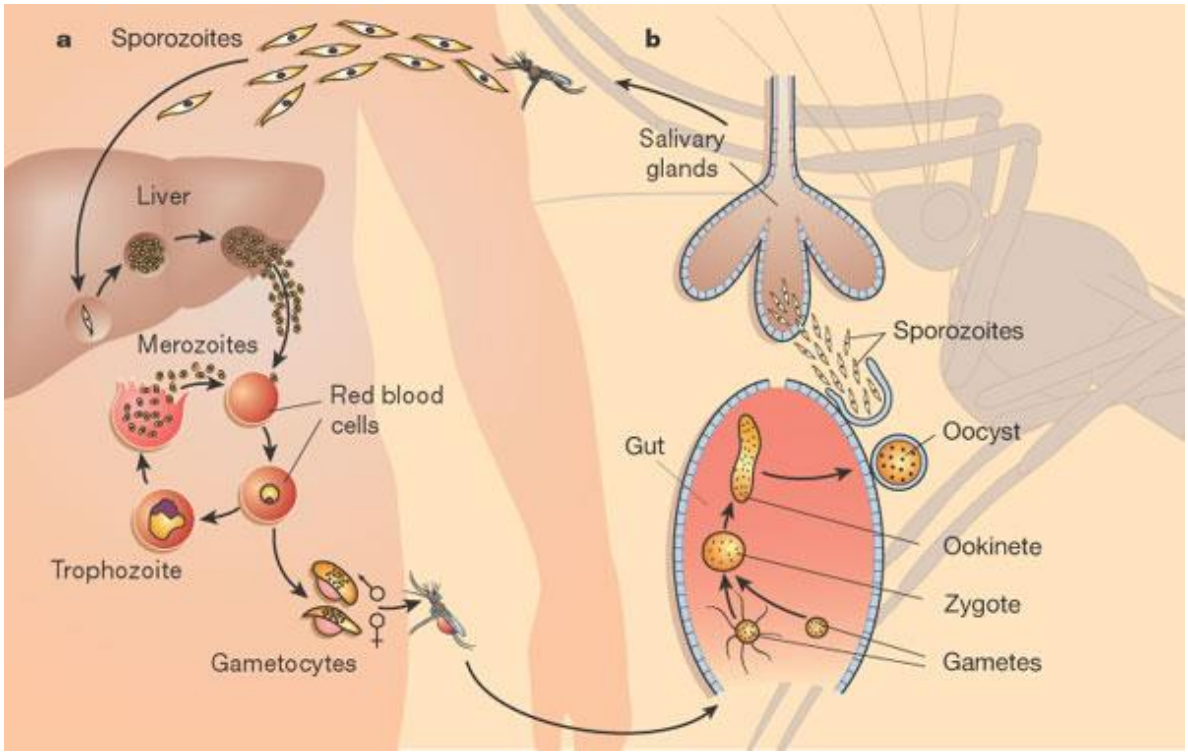

Figure 1. The life cycle of *Plasmodium falciparum* (Adopted from Wirth, 2002 and reproduced with permission from Nature Publishing Group)

1.4 THE DISEASE

Clinical malaria is primarily characterized as a febrile disease although, affected individuals can display numerous non-specific symptoms resembling those of common viral infections. Common symptoms include dizziness, malaise, myalgia, abdominal pain, nausea, vomiting, mild diarrhea, and dry cough. Physical signs include fever, jaundice, pallor, orthostatic hypotension, hepatomegaly, and splenomegaly. The infection may also pass unremarkable, even without fever and is then referred to as an asymptomatic infection.

1.4.1 Severe malaria

Severe malaria is almost exclusively caused by *P.falciparum* and mostly affects young children. It is a complex disease that can affect multiple organs and the clinical manifestations can appear alone or in combinations. Complications involve the nervous, respiratory, renal, and/or hematopoietic systems [31].

Clinical symptoms in severe malaria may include:

- Hyperparasitemia (>2% infected RBC)
- Cerebral malaria with seizures or coma
- Metabolic acidosis
- Acute respiratory distress
- Severe anaemia (Haemoglobin <5g/100mL)
- Hypoglycaemia (<2.2mmol/L)
- Pulmonary oedema
- Acute kidney injury
- Jaundice

The manifestations of severe malaria depend on age. Severe malaria in children mainly involve three clinical syndromes: cerebral malaria, metabolic acidosis and severe anemia caused by increased splenic removal of red blood cells and the destruction of red blood cells during parasite egress [32] [33]. In adults cerebral malaria is the most common severe manifestation and cause of death (WHO). Despite treatment, with the best acting drugs cerebral malaria and metabolic acidosis have a mortality rate of 15–20%, and survivors may suffer from persistent neurological disorders. Without appropriate treatment, clinical malaria can, within hours, progress from an uncomplicated to a severe disease state with lethal outcome without appropriate treatment. The greatest predictors of a lethal outcome is coma and metabolic acidosis and the incidences of these outcomes are similar at all ages [34]. *P. falciparum* is also the cause of pregnancy associated malaria (PAM) which affects both the mother and the infant.

1.4.1.1 Cerebral Malaria (CM)

One of the most lethal and severe neurological complications of malaria is cerebral malaria (CM). It is clinically defined as unarousable coma with the exclusion of all other causes and detectable Plasmodium falciparum parasites on peripheral blood smears (WHO, 2000). CM

is a complex multi-organ dysfunction that involves several complications. Main clinical features are coma, seizures, respiratory distress and retinopathy [35]. CM has a fatality rate between 15-20% and surviving patients may have sustained significant brain injury [36] [37]. The mechanism leading to brain injury is poorly understood as well as why some patients have poor outcomes and others may fully recover. The crucial pathophysiological cause for the pathogenesis is due to parasite sequestration in the cerebral microvasculature and an imbalance between pro-inflammatory and anti-inflammatory mediators [38] Marchiafava and Bignami, 1990. Parasites sequestration leads to extensive micro vascular obstruction that impairs the blood flow through the tissue, which may lead to hypoxia and eventually to coma. Immune host cells are recruited to the site of the sequestration and a widespread endothelia activation occur releasing factors that may lead to the loss of integrity of the blood brain barrier [39] [40]. It is clear that it is multi-factorial events that lead to CM, although the sequential order of events is poorly understood.

1.4.1.2 Pregnancy Associated Malaria (PAM)

During pregnancy, specifically in the first and second women are more susceptible to malaria. This is due to the sequestration of infected erythrocytes in the placenta. The Chondroitin Sulphate A (CSA) is a sugar that is expressed on the syncytiotrophoblast cells in the placenta in high numbers. A sub population of parasites express a specific surface antigen known as the VAR2CSA variant of PfEMP1 that specifically binds to CSA which leads to accumulation of parasites thus restricting blood flow and nutrient passage across the placenta [41] [42]. Pregnancy associated malaria (PAM) results in increased risk of maternal anemia, newborn anemia, infant low birth weight, still-birth and premature birth. Between 100 000 - 250 000 infant and fetal deaths and 1500 maternal deaths are caused each year by PAM [43].

1.5 IMMUNITY TO PLASMODIUM FALCIPARUM MALARIA

Acquired immunity to *P.falciparum* malaria is a complex process that is only partially understood. Sterile immunity is probably never achieved to parasites but complete protection from severe disease and death is acquired after multiple infections, this is why humans with no previous encounters to malaria and children living in malaria endemic countries are more vulnerable to severe disease and death [44]. In humans living in endemic areas of *P.falciparum* transmission the development of immunity is a relatively slow and gradual process. Children below the age of 3 to 4 months are protected against malaria. The complete mechanism of this protection is not understood although important roles for the

passive transfer of maternal IgG and IgA and possibly lactoferrin and fetal haemoglobin have been suggested [45] [46] [47] [48] [49]. Depending on the transmission intensity, children at the age of 2 to 5 years start to acquire protection against severe disease, which can gradually develop into full protection against symptomatic infection. Although, interruption of exposure can also lead to loss of protection which implies that the immunity is short-lived and there is a need for repeated infections over the life time of an individual to maintain protection [50].

It is believed that both the innate and the adaptive immune mechanism contributes to protection, the innate system functions to limit the maximum parasite density, and the gradually acquired adaptive mechanisms involving antibody response to several parasite antigens is required for complete parasite elimination [51] [52] [53]The possible mechanism of the adaptive immunity is many, including antibodies that block invasion of sporozoites into liver cells, antibodies that block invasion of merozoites into erythrocytes [54] and antibodies that prevent sequestration by binding to parasite adhesion molecules and facilitate parasite clearance [55] [56].

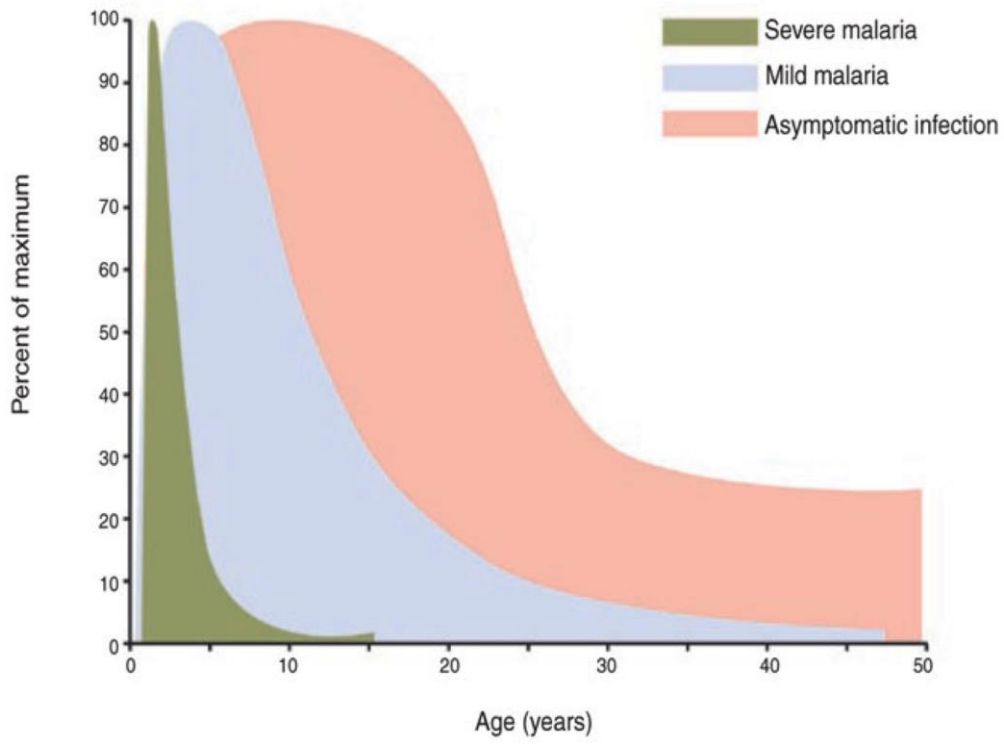

Figure 2. Acquisition of immunity to malaria by individuals living in endemic areas. (Adopted from Langhorne, 2008 and reproduced with the permission from Nature Publishing Group).

1.6 TREATMENT AND DEVELOPMENT OF A VACCINE

The earliest effective treatment of malaria came from extracts of the Chinchona bark tree that was originally used by the Quechua people of Peru, Bolivia and Ecuador to treat fevers and symptoms of malaria. The basic structure of the active metabolite, quinoline leads to the formation of a group of synthetic antimalarial including chloroquine (CQ), amodiaquine, piperaquine, mefloquine and primaquine. All acting on slightly different blood stages of the parasites and not on all Plasmodium species. CQ has been used extensively since 1947 for treatment and prevention of malaria [57]. QC and the related compounds act by inhibiting the formation of hemozoin (crystals converted by the parasites from the toxic produced during haemoglobin digestion), which leads to toxification of the parasite. *P.falciparum* resistance to CQ was first reported from the Thai–Cambodian border regions in 1957 and shortly after on the Colombian–Venezuelan border today it has spread to all of sub-Saharan Africa and most parts of the world [58] [59] [60]. CQ is still effective as treatment for most malaria caused by *P.vivax, P.ovale* and *P.malariae.*

As extracts from the Cinchona bark tree was used in South America to treat fevers the leaves of Artemisia *annua* was instead used in China for over thousand of years. Artemisinin also known as qinghaosu is an antimalarial isolated from the plant *Artemisia annua*, or sweet wormwood. Last years Nobel prize 2015 in Medicine was partly awarded to Youyou Tu (China) for her discoveries to successfully extract the active component from Artemisia annua. Today artemisinin derivatives such as artesunate and artemether are the first line treatment to patients suffering from severe malaria (WHO guidelines 2015). It is active against all Plasmodium species and acts rapidly reducing parasites in the blood. The mechanism of action is not fully understood and the effect on mature gametocytes is limited [61], why several of the de novo drug efforts aim to target the late gametocyte development stages, in order to find a way to prevent transmission of the disease.

Recommended by the WHO as the first line treatment for uncomplicated malaria cases is an artemisinin-based combination therapy (ACT) that combines artemisinin derivatives with a partner drug. Other available treatments are the antifolate compounds such as sulphadoxine and pyrimethamine, which are used in combination (SP) due to the resistance of parasites when they are used as mono therapy. The antifolates act by inhibiting parasite enzymes necessary for folate biosynthesis and inhibit the synthesis of nucleic acids and DNA synthesis [60].

Currently artemisinin resistance has been confirmed in 5 countries of the Greater Mekong sub region (GMS): Cambodia, the Lao People’s Democratic Republic, Myanmar, Thailand

and Vietnam (WHO). A recent alarming study showed multidrug tolerance arising from artemisinin resistant parasites *in vitro*. Due to the ability of the parasites to go into drug-induced quiescence they can survive exposure to several unrelated antimalarial drugs [62]. This is a novel resistance pattern that may be a major threat if it is happening *in vivo*.

1.6.1 Sevuparin as an adjunctive therapy

Severe falciparum malaria is a medical emergence and needs immediate diagnosis and an effective treatment. Sevuparin, Dilaforette´s lead drug candidate is currently being tested in a multi-center, double-blind, placebo-controlled phase II study, enrolling hospitalized Sickle-Cell Disease (SCD) patients experiencing vaso-occlusive crises (VOC). It acts as an anti-adhesive and *in vitro* studies in *P.falciparum* also shows that sevuparin blocks the merozoite invasion and inhibits adhesion to endothelial cell and binding to uninfected erythrocytes phenomena's known as *sequestration* and *rosetting* described in sections 1.8.3 - 1.8.5. Additionally, in animal models of severe malaria, sevuparin reduces the sequestration of infected erythrocytes in the microvasculature [63] [64].

1.6.2 Vaccine development

Currently, there is no malaria vaccine available. Although there are over 30 vaccine candidates currently tested in clinical trials or in advanced preclinical trials. The development of a malaria vaccine has mainly focused on preventing disease from *P.falciparum* and to a lesser extent, on *P.vivax*. The most advanced candidate is the RTS,S /AS01 vaccine, targeting sporozoites, that has completed phase three trials in African children the efficacy was 27%-39% in those children that received a fourth dose (Malaria vaccine: WHO position paper – January 2016). Several proteins located on the surface of the merozoite are among the candidates for vaccines and also as drug targets for inhibiting invasion. Some showing partial efficacy in clinical trials in humans [65].

1.7 HUMAN GENETICS SHAPED BY PLASMODIUM

Malaria is the major infectious disease that has shaped the human genome. In general, genetic mutations that deform the red blood cells has persisted and spread in many regions of the world where the population at some time has been affected by malaria. The geographic distributions of several traits that protect against severe malaria as sickle cell trait, ovalocytosis, thalassaemias, haemoglobins C and E, and glucose-6-phosphate

dehydrogenase (G6PD), are similar to that of malaria as well as the Duffy blood group negative and the ABO blood group distribution [66]. In the case of sickle cell disease (haemoglobin AS (HbAS)) a single point mutation turns normal blood cells into rigid, frozen crescents called sickle cells. A new-born homozygote will not survive childhood without modern medicine while the heterozygote is protected against cerebral malaria and severe malarial anaemia [66, 67] [68]. In ovalocytosis the red blood cells are oval and so rigid that they reduce invasion of the parasites. Haemoglobins AC (HbAC) and CC (HbCC) reduce parasite cytoadherence, haemoglobin AE (HbAE) reduce parasite multiplication at high densities and G6PD deficiency reduce parasite densities by disrupting the enzyme G6PD needed for cellular oxygen damage repair. The sickle cell trait has spread through Africa, South Asia and the Middle East, haemoglobin E spread throughout South-East Asia [69] thalassemia in the Middle East and Mediterranean, ovalocytosis throughout the Pacific region [70].

1.7.1 The ABO blood group and malaria

The ABO blood group was discovered by Karl Landsteiner in 1901 and is the most widely used blood group system and most important in transfusion medicine. An individuals blood group is determined by the antigens expressed on the red blood cell. These antigens can be either sugars or proteins and are attached to various components of the membrane including membranes of platelets and endothelia. The ABO blood groups are oligosaccharides attached to glycoproteins and glycolipids that project from the membrane of the RBC. The Czech serologist Jan Janský classified them into four blood types A, B, AB and O depending on the antigens they express. A single gene that encodes a glycosyltransferas, an enzyme that modifies the carbohydrate content of cells controls the blood type of an individual. The so called ABO locus, has three main allelic forms - A,B and O. The O antigen is the precursor ABO antigen found in all the three blood types, also referred to as the H antigen with the form (—Lipid—Glucose—Galactose—*N*-acetylglucosamine—Galactose—Fucose). The O allele encodes an inactive glycosyltransferase. The A allele encodes a GalNAc transferase that adds the extra N-acetylgalactosamine to the H antigen producing the A antigen while the B allele encodes a Gal transferase that adds the extra galactose creating the B antigen. The A and B blood group also have subtypes defined by the level of either A-antigen or B-antigen expressed on the surface

For example, the A1 subtype express approximately five times more A antigen on the surface of the red blood cell than the A2 subtype [71]. The function of the ABO blood groups is not known. Although, the distribution of different ABO blood types in populations around the world suggests that specific blood types may have been evolutionary selected for and that the selection is pathogen-driven by the advantaged e.g. resistance against an infectious disease [72] [73]. Several associations have been made between particular ABO blood types and increased protection or susceptibility to a specific disease [74]. Such as, influencing the susceptibility to different pathogens responsible for disease e.g. severity of malaria and cholera [75] [76], *H.pylori* infections [77] and norovirus infections [78] [79]. Blood group O individuals also tend to have decreased levels of the von Willebrand Factor, a protein in the blood involved in blood clotting [80]. Worldwide blood type O is most common then A and B while AB is the least common. In malaria several studies have suggested a correlation between blood group and the severity of disease where blood group O individuals would be protected against severe disease compared to individuals of blood group A, B or AB. One of the major pathological features caused by P.falciparum is the ability of the parasites to sequester in the microvasculature and to rosette, a phenotype where the infected red blood cell binds to three or more uninfected red blood cells which is described in more detail in sections 1.8.3 - 1.8.5. A study by Rowe et al. showed that individuals with mild disease were more likely blood group O and individuals with severe disease were more likely blood group A or B. A following study proved that this protection was due to reduced rosetting in children with blood group O [81] [82].

Several data taken together imply that malaria has had en strong evolutionary impact on the global distribution of the ABO blood groups in the human population [83] [84] [85] [86].

1.8 PLASMODIUM FALCIPARUM PATHOGENESIS

1.8.1 Erythrocyte invasion

Importantly, *P.falciparum* has a broader repertoire of infecting different stages of RBCs compared to other *P spp*, which adds greatly to the elevated level of virulence found in *P.falciparum.* Merozoite invasion is a multi-step process that is dependent on the sequential contact between parasites proteins on the surface of the merozoite and specific ligands on the red blood cell. The process of invasion can be divided into three distinct stages; pre-invasion, internalization and echinocytosis first described in *P.reichenowi* by Dvorak et al.,

1975 [87] and recently monitored on film by Weiss et al., 2015 [88] where a probable order of the sequentially acting receptor-ligand interactions is shown. All three stages happen within a minute; within the first 10s the pre-invasion step occurs categorized by a confound deformation of the target erythrocyte. Followed by a 20-60s internalization step and echinocytosis which is when the red blood cell membrane appears with spiky protrusions, thereof the Greek name *echinos* meaning `hedge hog´ or `sea-urchin`. This appearance remains for 5-10 minutes before the erythrocyte returns to the normal bio-concave shape.

By using many different invasion ligands *P.falciparum* can invade any type of erythrocyte in contrast to *P.vivax* where the loss of the Duffy blood group receptor the parasites cannot invade. At least a dozen receptor-ligand interactions are known to be important for the invasion of the erythrocyte by the *P.falciparum* merozoites [88]. In the initial attachment during the pre-invasion the merozoite can bind the target erythrocyte in any direction in a low affinity interaction. This interaction possibly occurs through glycosylphosphatidylinositol (GPI) anchored merozoite surface proteins (MSPs) which are the major component of the merozoite surface coat [89] [90]. The most abundant protein is the MSP1 which is considered to be essential for the initial contact and for parasites survival it is also a major vaccine candidate [87] [91] [92] [93].

After the initial contact the merozoite reorients itself and interacts with the erythrocyte through its apical end and forms a tight junction releasing proteins from the micronemes and rhoptries. Proteins involved in the invasion process belong to the erythrocyte binding antigen family (EBAs), and the reticulocyte-binding like homologs (PfRh) [94] [95] [88]. With the exception for two of the PfRh proteins that seem to have distinct functions the remaining proteins seem to be dispensable with slight overlapping functions and with the ability to compensate for the lack of another. The PfRh1 shows a distinct function of signalling the release of micronemes containing EBA-175 and PfRh5 specifically interacts with the erythrocyte protein basin necessary for parasite invasion [96] [97].

The PfRh5-basigin interaction is recently suggested to mediate an open connection between the apical tip of the parasite and the host cell immediately prior to invasion that serves as a conduit for invasion proteins forming the tight junction [88]. A key component of the tight junction is the apical membrane 1 antigen (AMA1) that is secreted onto the surface of the merozoite upon egress from the schizont. AMA1 interacts with rhoptry neck protein 2 (RON2) that is secreted into the RBC membrane with a small extracellular loop that interacts with AMA1 [98] [99] [100] [101]. During final invasion the tight junction moves from the apical to the posterior end of the merozoite. At the same time the coat covering the merozoite containing invasion proteins are removed in a process known as ”shedding”. This

involves a serine protease called SUB2 [102]. When the merozoite is fully inside the RBC the parasitophorous vacuole formed during invasion encloses it in a physical barrier between the parasite and the host cytosol, in which it resides during the intra-erythrocytic development.

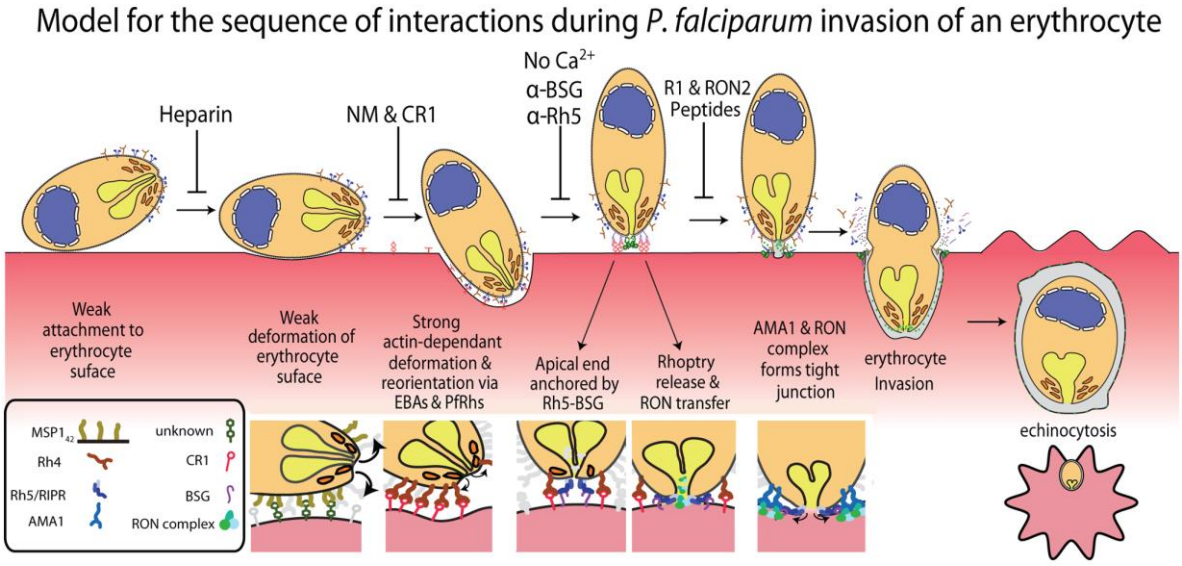

Figure 3. Sequence of interactions during *P.falciparum* invasion of an erythrocyte (Adopted from Weiss, 2015 and reproduced with permission from PLOS)

1.8.2 Remodelling the host cell

For the parasite, the red blood cell is an intracellular ”hideout” with a major source of nutrient in the form of haemoglobin. However, the mature red blood cells have no nucleus, no machinery for protein synthesis nor a molecule-trafficking machinery, so the parasite has to modify the host cell and provide these these vital mechanisms on its own. The uninfected erythrocyte is highly deformable with an elastic membrane and cytoskeleton. When it becomes infected with *P.falciparum* it looses its deformability, becomes rigid and the cell permeability and adhesiveness increases [103] [104]. For the parasite to successfully invade the red blood cell, the merozoite must first transiently disrupt the blood cell membrane. Inside the red blood cell, the parasite develops in its own compartment, the parasitophorous vacuole (PV) surrounded by the parasitophorous vacuole membrane (PVM) that is initially formed during invasion as an invagination of the red blood cell surface. The PVM consists of the infected RBC membrane lipid raft proteins and parasite-

coded proteins such as the early-transcribed membrane proteins (ETRAMPS) [105] [106] [107] [108] [109]. In the early hours after invasion the parasite starts to remodel the RBC through extensive physiological and structural modifications to promote its own proliferation. The first modification and the basis for parasite survival in the RBC is the export of proteins to different cellular destinations where they are needed in the cytosol and to the RBC membrane. To enable the trafficking of virulence proteins, lipids and other molecules the parasite generates membranous networks such as the tubovesicular network that emanate from the parasitophorous vacuole and expands into the erythrocyte cytoplasm [110] and structures called the Maurer´s clefts (MC). These structures were described already in 1902 by Maurer as spots in the RBC cytosol beyond the parasite´s PV [111] [110]. The MCs are donut shaped organelles with a translucent lumen and electron-dense coats detected in the RBC cytosol from the early ring stage (2-6h) and onwards [112] [113] [114]. The MCs are thought to be an intermediate compartment for sorting and exporting proteins from the parasite to the RBC membrane [115] [116] [117] [118] [117] [119]. Later in the cycle, before merozoite egression these structures seem to actively disassemble [120].

The *P.falciparum* `exportome` include all the proteins that are exported from the PV and comprise approximately 10% (550 proteins) of all predicted open reading frames in the parasite. These are classified into two groups. The larger group contains a five amino acid signal sequence called the *Plasmodium* export element (PEXEL), localized approximately 20-30 amino acids after the first signal sequence that promotes entry into the parasite endoplasmic reticulum (ER) [121] [122] [123]. The second smaller group called the PEXEL-negative exported proteins (PNEPS) lack a PEXEL motif and a defined motif but contain a hydrophobic region that mediates export [124] [125] [123]. In order to reach the host cell cytoplasm, the exported proteins need to cross three membranes. The first is the ER parasite membrane within the parasite where the PEXEL motif acts, the proteins the leave the parasite and enters the host cell first by crossing the parasite plasma membrane (PPM) and second the PVM. They cross the PVM via a protein transport translocon, named the *Plasmodium* translocon of exported proteins (PTEX), which is a complex that forms a protein export channel in the PVM essential for passage of all type of proteins into the host cell and also essential for blood-stage growth [126] [127] [128].

The exact route of transportation for surface antigens through the host cell cytosol is not known although an increasing number of the exported proteins are transiently associated to

the Maurer´s clefts. Alternative transportation pathways utilizing vesicles or transported as soluble intermediates have also been suggested [129] [130].
Proteins important for MC formation and which localize permanently at the MC structures are the skeleton binding protein 1 (SBP1) that attaches the MC to the cytoskeleton, the membrane-associated histidine rich protein 1 (MAHRP1), the ring exposed protein 1 (REX1) that sculptures the structures of MC, *P.falciparum* erythrocyte membrane protein 3 (PfEMP3) and Pf332 that binds to spectrin, the cytoskeleton and increases the rigidity of the infected RBC [110].

Another significant modification of the host cell membrane is the reorganization of the cytoskeletal network by parasite-induced actin remodelling, which dramatically increases the stiffness of the cell and the formation of knob-like protrusions a few hours after the cell is infected [131] [132]. The knobs are electron dense protrusion at the RBC surface detected a few hours after the cell is infected and with a diameter of approximately 100nm [133]. These serve as cytoadhesion attachment points by anchoring the surface exposed PfEMP1 molecule to the host cytoskeleton. A key protein of the knobs is the knob-associated histidine-rich protein (KAHRP) [134]. KAHRP is present on the cytosolic side of the membrane and may interact with spectrin, actin and ankyrin in the cytoskeleton and the intracellular acidic terminal segment (ATS)- domain of the PfEMP1 proteins [135] [136]. Its presence is also associated with stronger cytoadhesion under flow [134].

1.8.3 Sequestration

One of the key pathological features of severe malaria is parasite sequestration in the microvasculature causing blood flow obstruction and inflammation in host organs. For the parasite this is likely a survival mechanism, by sequestering it avoids to be washed away in the bloodstream recognized and destroyed by the spleen. The parasite expresses molecules on the surface of the infected red blood cell making the otherwise smooth cell `sticky`. The infected cell attaches to different receptors on endothelial cells lining the blood vessels a phenomenon known as *cytoadhesion* or to other uninfected red blood cells called *rosetting* [137] [85] [138]. The ”stickiness” is mediated by the expression of different parasite antigens on the surface of the red blood cell. As early as 1894 the Italian malariologists Marchiafava and Bignami saw when they examined blood samples from patients that, only young parasites, the, so called ring-stages could be seen in the microscope (Marchiafava and Bignami, 1894). This is due to the fact that the more mature stage parasites express surface adhesins and are sequestered in the post-capillary venues of various tissues and

organs and withdrawn from the blood circulation. The consequences of the sequestration can be dire. For example, massive sequestration in the microvasculature of the brain is the major pathophysiological feature of cerebral malaria, which is the main cause of death from malaria infection. Parasites expressing the so-called PfEMP1-VAR2CSA ligand sequester in the placenta of pregnant women by binding to the CSA receptor resulting in the accumulation of parasites in the placenta, causing severe clinical consequences for the mother and her unborn baby [139].

The sequestration is explained by phenomena known as: cytoadhesion, rosetting, auto agglutination and platelet-mediated clumping. The characteristics of these phenomena including host receptors and parasites ligands involved are described in more detail in the following sections.

1.8.4 Cytoadhesion

Cytoadhesion is the binding of infected red blood cells to micro vascular endothelial cells in various organs such as the brain, placenta, intestine, lung and liver. For example, the binding of the parasite antigen *plasmodium falciparum* erythrocyte membrane protein1 (*Pf*EMP1) to protein and carbohydrate ligands on the endothelial cell surface. These ligands include the thrombospondin receptor CD36 [140], intercellular adhesion molecule 1 (ICAM1) [141], platelet/endothelial cell adhesion molecule (PECAM), complement receptor 1 (CR1), heparin sulphate and chondroitin sulphate A (CSA) and EPCR also known as activated protein C (APC) receptor [142] [143].

1.8.5 Rosetting

The rosetting phenomena is characterized by the binding of one infected RBC to two or more uninfected RBCs creating a flower shaped cluster of cells. In experimental models the formation of rosettes leads to micro-vascular obstruction [63] [144]. It is commonly associated to disease severity as seen in human autopsies [145] [32] [146] and linked to severe pathogenesis such as cerebral malaria and malarial anemia [147] [148]. Rosetting is seen once the antigens involved in this specific adhesion are expressed on the red blood cell surface. This occurs in the trophozoite stage of the intra erythrocytic developmental cycle.

The rosetting phenomenon was first detected in the microscope in laboratory strains *in vitro* but were later also seen in fresh clinical isolates obtained from malaria infected patients [149] [150] [147]. Importantly, many isolates quickly loose their rosetting phenotype once

they are adapted to *in vitro* conditions, likely due to the down regulation of surface antigens upon lab adaption.

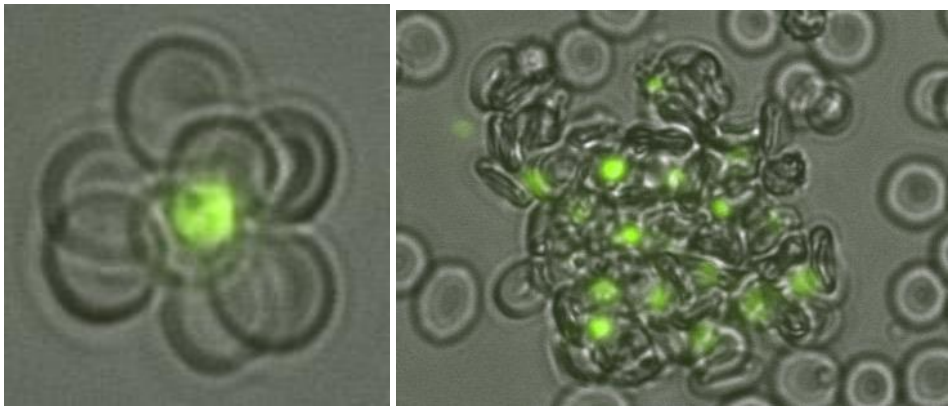

Figure 4. Rosette of the FCR3S1.2 parasite clone (left) and a giant rosette of the 3D7P2G12 tDT-164 clone with trophozoites (in green) binding to several uninfected RBCs (right)

Clinical isolates and laboratory-adapted strains vary in their degree of rosette formation and not all parasites form rosettes but those that do contribute to the severity of the disease. This has been shown in several studies in African children where the ability of the parasite to rosette and severity of malaria was correlated [147] [151] [152] [153] [81]. The mechanism by which rosetting contributes to disease severity was further correlated to high parasitemia loads [154] [155]. A number of receptors on the red blood cell surface have been identified to mediate rosette formation including serum factors and parasite antigens. The parasite adhesins known to date are the PfEMP1 proteins, member of the group A *var* genes [156] [157] [158], the A RIFIN members of the *rif* gene family [159] and the STEVOR proteins [160]. These are discussed in more detail in section 1.8.3, 1.8.4 and 1.8.5.

The diameter of a micro vessel is in the order 100 µm and below and the diameter of a red blood cell is approximately 5µm so it is easy to understand that formation of several giant rosettes (diameter 10- 50 µm) can clog the blood vessels. However, if it has any more specific function for the parasites than to avoid passage through the spleen is not as clear. A positive correlation between the peripheral parasitemia rate and the level of rosetting has been shown so it is hypothesized to facilitate the reinvasion of new erythrocytes [161]. Although, no *in vitro* studies have proven an increased invasion, since non-rosetting parasites grow and multiply as well as rosetting parasites in the lab, neither does it appear to protect merozoites from invasion inhibitory antibodies [162] [163]. As Hviid et al., and Wang put it ”it may simply be a marker of adhesion to endothelial host receptor motifs also

present on erythrocytes" [164]. Although, recently we observed that tight rosettes as those formed in blood group A masks the iRBC from detection by antibodies to the PfEMP1 ligand in *in vitro* cultures. This suggests that tight rosettes as those formed in blood group A may also act as an immune evasion mechanism protecting the parasites from antibodies to the major ligand, PfEMP1 attacked by the immune system (**Paper II)** [159]. Erythrocyte receptors mediating rosetting appear to be mostly carbohydrates [165] [166] [167] such as the ABO blood group antigens. Other receptors on the red blood cell implicated in rosetting include the complement receptor-1 (CR1), blood group A (BgA), heparin sulphate (HS) and CD36.

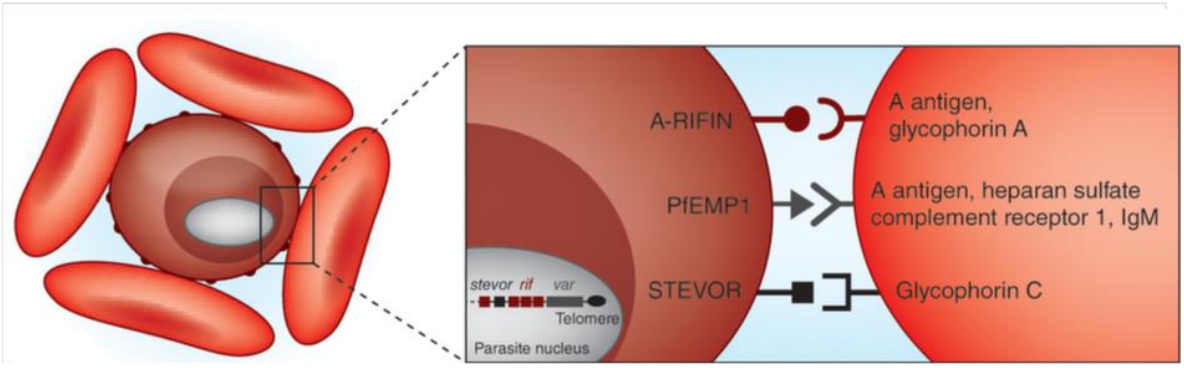

Figure 5. Mediators of rosetting. (Adopted from Mancino-Silva & Mota, 2015 and reproduced with permission from Nature Publishing Group)

1.8.5.1 Complement receptor 1

CR1 is a membrane glycoprotein present on the surface of RBC, monocytes, granulocytes, dendritic cells, B cells and T cells. Rowe et al showed that CR1-deficient RBCs were not able to form rosettes with laboratory strains and this was later confirmed with a monoclonal antibody against CR1 that showed reverse rosette in clinical isolates from Africa [157] [168].

1.8.5.2 Blood group A

The blood group A antigen is a sugar present on the RBC membrane in individuals with blood group A. Rosetting *P.falciparum* isolates show a preference of binding to A RBCs while forming smaller and looser rosettes in blood group O [169] [170]. It has also been shown that fresh isolates of P.falciparum from children with severe malaria in Kenya bound the A antigen more avidly than strains from children with mild disease [171]. In general, rosette formation has been shown to preferentially involve blood group A, B or AB in

different studies of African strains and with a significant reduction of rosettes in blood group O individuals [172] [165] [170] [81].

Heparan sulphate (HS) is present on the RBC surface and mediates rosetting with certain *Pf*EMP1 molecules and specifically with the N terminal region of the protein. Earlier studies show that heparin, a highly sulphate version of HS and heparin derivatives can disrupt rosettes [170] [166] [173] [174].

1.8.5.3 Serum factors

Most parasite strains studied require the presence of serum to form rosettes [169]. Serum factors such as non-immune IgM is shown to have an important role in the formation of rosettes both in laboratory strains and in patients isolates [175] [169] [162] [176]. Earlier observations showed fibrillar material between the IE and the surrounding RBC containing IgM, suggesting that IgM and other serum factors can serve as `bridges` between the IE and the surrounding erythrocytes [175]. Although the presence of IgM is not prerequisite for rosetting [177], since it is also detected in non rosetting parasites [178]. Recent data shows that the binding of IgM and the serum protein $\alpha_2$macroglobulin bind to multiple Plasmodium falciparum erythrocyte membrane protein 1 (PfEMP1) molecules on the surface of the IE holding them together as a `bouquet` which increases the avidity for receptors on the surrounding RBCs [179] [180]. In placental binding parasites strains the IgM molecules seem to mask the IgG epitopes on the PfEMP1 molecules interfering with the phagocytosis of IgG-opsonized IEs [181] [182]. Other serum proteins such as von Willebrand factor, Complement factor D, serum albumin and fibrinogen also seem to have a role in rosette formation but exactly how they act is not known [183] [169].

1.8.5.4 Platelet mediated clumping

Infected RBCs that bind to other infected RBCs form so called auto agglutinates that can involve a large number of infected cells. Auto agglutination is mediated by platelets and the expression of the platelet surface glycoprotein CD36 [184] [185].

The auto agglutinating phenotype has also been associated with severe malaria and is a common adhesion phenotype observed in laboratory strains and in isolates [186] [187] [147] [81] [184].

1.9 ANTIGENIC VARIATION

Antigenic variation is the ability of infectious organisms to alter their molecules exposed to the immune system of their host and by doing this they avoid antibody recognition. Many pathogens including viruses [188], bacteria [189], fungi [190] and protozoan parasites have evolved this mechanism to sustain infection and survive in the host. The virulence and mortality of *P. falciparum* infection is directly linked to the ability of the parasite to persist and proliferate in the human host during the blood stage infection. To escape from the host´s immune system and avoid elimination by the spleen the parasite has developed mechanisms such as *rosetting* and *sequestration* (previously described) and *antigenic variation*. By expressing different repertoires of exposed parasite antigens on the erythrocyte surface, the adaptive immune system fails to generate novel Abs at high enough turnover rate to recognize and destroy all parasites. The genes involved in antigenic variation are highly polymorphic and members of multi copy gene families that the parasite can switch expression between and generate a substantial antigenic diversity to persist in the host.

1.9.1 Variant surface antigens

Parasite molecules expressed on the surface of the infected erythrocyte are referred to as variant surface antigens. These include the *plasmodium falciparum* erythrocyte membrane protein 1, PfEMP1, repetitive interspersed family, RIFIN, the surface associated interspersed family, the sub telomeric variable open reading frame STEVOR and the surface associated interspersed family SURFIN. They are encoded by mutlicopy gene families and are involved in antigenic variation, immune evasion, and host sequestration and are important targets of acquired protective immunity. The *Pf*EMP1, *plasmodium falciparum* erythrocyte membrane protein 1, encoded by the *var* gene family is the most well characterized variable surface antigen.

1.9.2 PfEMP1

The plasmodium falciparum erythrocyte membrane protein 1, PfEMP1 is a family of high molecular weight proteins (approximately 200-450kD) encoded by approximately 60 different *var* genes in the haploid *P.falciparum* genome [191]. Their main function is to mediate sequestration of infected erythrocytes and they contribute to disease severity by binding to specific tissues as seen in human autopsy samples and enabling the parasite to evade host immunity. They were first described as parasite antigens displayed in protrusion

like extensions on the surface of the RBC and involved in tissue specific sequestration [192] [193] [194] [192] [195]. And showed by Leech et al., that they were high molecular weight proteins by surface iodination experiments [196]. It was in 1988 they were officially named PfEMP1 by Howard et al [197]. Ten years later three research groups using different experimental approaches came to the same conclusion that PfEMP1 was actually a family of several proteins and the genes that encoded them were termed the *var* genes [198] [199] [200]. The *var* genes are transcribed at the ring stage (3-18 hours post invasion, hpi) [201] [202] and *Pf*EMP1 proteins are detected on the surface of the IEs as the parasite reaches the early trophozoite stage. All of the *var* genes have a two-exon structure. Exon one is hyper variable in sequence and encodes the extracellular part of the protein and exon two is conserved and encodes a short trans membrane domain and an intracellular acid terminal sequence (ATS) that anchors the protein to the host cytoskeleton. The extracellular part of the protein is built of an N-terminal segment (NTS) and due to the high sequence variability an array of different subtypes of Duffy binding-like domains (DBL) and cysteine rich interdomain regions (CIDR) [203] [204] [205]. Despite their extreme diversity the *var* genes can be divided into four different groups depending on their chromosomal location, upstream promoter sequence (*ups*) and the direction of transcription. In the P. falciparum 3D7 genome the var repertoire consists of 10 Group A *var*, 22 Group B, 4 Group B/A , 13 Group C and 9 Group B/C *var* genes. Most of the *var* genes belong to the Group A and B and are located in the subtelomeric regions adjacent to the telomere associated repeat (TARE). The Group A genes are transcribed towards the telomere while Group B and B/A towards the centromere. The 13 Group C and 9 B/C var genes are found in the internal chromosomal regions [191] [206] [207]. The structural characteristics of different PfEMP1s defines their receptor specificity, an individual PfEMP1 may also contain different adhesive domains with different receptor specificity which can allow the binding to several host cell receptors [208] [209] [210]. Each *var* gene typically comprises 4 to 7 DBL and CIDR domains, each domain being subdivided in various classes. The DBLs have been classified into six different types (alpha, beta, gamma, delta, epsilon,) and the CIDR into five types (alpha, beta, gamma, delta and pam) [207]. Certain combinations of DBL and CIDR domains tend to occur together and these have been classified into specific domain cassettes (DCs) that are linked to different adhesive phenotypes. The DC4 is a cassette composed of three domains ($DBL\alpha_{1.1/1.4}$-$CIDR\alpha_{1.6}$-$DBL\beta_3$) and defines a subfamily of Group A PfEMP1 that mediates binding to ICAM-1 (CD54) but not to CD36 [211]. The DBLb3 domain of these PfEMP1s is predicted to contain the binding site for ICAM-1 on the endothelial cell surface [212] [213] [214]. The DC5 cassette present in a Group A subfamily mediates

adhesion to PECAM-1 [215] [216] present on the surface of endothelial cells, monocytes, platelets and granulocytes [198]. The PfEMP1 containing DC8 and DC13 found in Group B and Group A PfEMP1 respectively are highly transcribed in children with severe malaria [217] and are found in IE sequestered in the brain through binding to the EPCR receptor [218] [219] [142].

PfEMPs involved in rosetting belong to the UpsA type var genes and the adhesive interaction is mediated by the semi-conserved, N-terminal head structure of PfEMP1 [220] [208] [157] [221]. The DBL1 alpha domain is the part of the protein that mediates rosetting and endothelial binding of iRBC through heparin sulphate, blood group A antigen, complement receptor 1 [220] [173] [170] [222] [223] and EPCR [142]. It is the most distal part of the PfEMP1 protein from the RBC membrane and the most conserved among the DBL domains [224] [207] [205].

1.9.3 RIFIN

The *rif* gene family (repetitive interspersed family) gene family is the largest identified multigene copy family in *P.falciparum* and is present as 150-200 copies per haploid genome [191]. Unlike the PfEMP1, the RIFIN proteins are small polypeptides (30-50 kDa) beginning with a putative signal sequence followed by a PEXEL (protein export element in *P.falciparum*) a semi conserved domain, a variable region and a conserved C-terminal domain [225] [226]. Traditionally they are described as two trans membrane (TM) proteins but recent topological studies indicate that they only have one TM domain near the C terminal end and that the semi-conserved N-terminal region is exposed to the outside of the erythrocyte surface [159] [227].

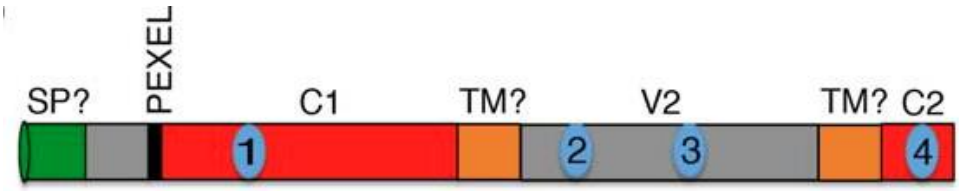

Fig 6. RIFIN protein structure; SP: signal peptide, C1: conserved domain 1, TM: transmembrane domain, V2: variable domain 2, C2: conserved C terminal domain. Circles 1-4 show the modified glycosylation sites to determine the localization of TM (Paper I)

The *rif* genes are located in clusters in the subtelomeric regions with neighboring *var* genes and are encoded by two exons. Bioinformatics analysis of the repertoire of RIFIN in the 3D7 parasite strain revealed two major subgroups within the family, group A and B type RIFIN which differ by a 25-amino acid insert in the semi conserved domain of A-type RIFIN. The majority of the RIFINs belong to the subgroup A (70%) [228]. During erythrocyte schizogony most *rif* genes are expressed at 12-27 hours post invasion (hpi) [229] [201] [202]. During the trophozoite stage the two subgroups appear to have different subcellular localizations while A RIFINs are exported to the erythrocyte membrane B RIFIN seem to retain inside the parasite cytosol [230] [115] [116] [231] [232] [228]. Similar, in merozoites A type RIFINs are associated to the apical end whereas B RIFINs are cytosolic. The expression of B type RIFINs has also been detected in gametocytes and sporozoites [231] [229] [231] [233]. Earlier studies show that A- type RIFIN proteins are transported via the Maurer's clefts to the erythrocyte membrane [231, 234, 235]. Recent confocal immunofluorescence analysis of the 3D7 parasite shows co localization of RIFIN with spectrin, skeleton binding protein (SBP1) and merozoite surface protein 1 (MSP1). Proteins localized at the erythrocyte plasma membrane, Maurer's clefts (MC) and merozoite surface respectively. In the MC, the major part of the protein is located inside the organelles [227].

The RIFIN N-terminal PEXEL sequence is subsequently cleaved by Plasmepsin V, which also cleaves the PEXEL motifs in STEVOR and RESA [236].

No function has been established yet to RIFIN expressed on gametocytes, sporozoites, and merozoites. Recently, A RIFINs expressed in CHO cells and a recombinant A RIFIN was shown to mediate binding to the blood group A antigen on blood type A RBCs and to sialic acid on glycophorin A of type O RBCs (**Paper I).**

Interestingly, expression of parasite adhesins on the iRBC surface has also been linked to sequestration in the rodent parasites *P.chabaudi* and *P.berghei* which lack *var* like gene families [237] [238] [239]. Common to all these parasites is the pir multigene family that codes for small variant antigens that have features similar to STEVOR and RIFIN [240] [241]. This suggests that the PIR proteins are adhesins which enable these parasites to rosette and sequester in the absence of PfEMP1.

In field isolates the expression of surface antigens including RIFINs have shown to be up regulated compared to lab adapted parasites strains [242] [243]. Specifically, in patients with cerebral malaria, A RIFIN proteins and specific subset of *var* genes that showed to be up regulated compared to asymptomatic patients [244] [245]. Additionally, no expression

of A-RIFINs (including other multi copy gene families *var* and *stevor)* was detected in parasites from a splenectomised patient suffering from clinical *falciparum* malaria whereas expression of B RIFINs was. Supporting the hypothesis that also the A RIFIN and STEVOR proteins are involved in the sequestration of parasites [246].

1.9.3.1 Antibodies to RIFIN

The role of RIFIN proteins in the development of immunity to clinical episodes of malaria is unclear. Since their discovery several studies have shown that RIFINs are targets of the human immune response [225] (Fernandez et al., 1999). During controlled *P.falciparum* infections in naive volunteers' antibodies to RIFIN were detected by Turner et al., in 2011 [247]. A study conducted in Lambaréné, Gabon also showed higher anti-rifin antibody levels in the majority of the adult population tested whereas the level of RIFIN antibodies was much lower in the malaria-exposed children [248]. In another study Gabonese children who were diagnosed with severe malaria and showed high antibody levels to RIFIN were also able to clear their parasites more efficiently than those with low antibody responses [249]. Recently, Tan et al discovered human monoclonal antibodies that were able to opsonize iRBC of different *P.falciparum* isolates by binding to members of the A-RIFINs. They achieved their broad reactivity through a large DNA fragment inserted between the V and DJ segments. The insert encodes the 98 amino acid collagen- binding domain of LAIR, an immunoglobulin superfamily inhibitory receptor. The antibodies binding to infected erythrocytes were produced from a B-cell clone carrying somatic mutations in the LAIR domain which abolished the binding to collagen and increased binding to RIFINs [250].

1.9.3.2 Rif gene expression and regulation

Like *var* genes the rifin have been shown to undergo expression switching and due to the high gene copy number and the localization of A RIFIN on the surface of the infected red blood cell suggest that they are involved in antigenic variation as PfEMP1 [226]. This was also supported by the transcriptional data of the rosetting parasite clone FCR3S1.2 showing one dominant expressed RIFIN, indicating an allelic exclusive expression pattern of *rif* genes at least from the same subgroup, similar to that of the *var* gene expression [159]. Few putative transcription factors have been identified in the Plasmodium genome [191], suggesting that other post-transcriptional mechanisms are important in regulating gene expression.

Epigenetic regulation through heterochromatin marks have been found to regulate the expression of clonally variant gene families located in the sub-telomeric regions of

chromosomes [251] [252]. In the case of the *var* genes the parasites can switch expression and this is a tightly regulated process but the exact mechnanistic is not fully understood. Epigenetic regulation by active epigenetic marks and modifying enzymes has been shown to correlate with the dominant var gene and facilitate clonal variant switching [251] [253] [254] [255]. And similar regulation of the expression of other gene families such as *rif* and *stevor* has been indicated [256]. It is suggested that antigenic variation and allelic exclusion may also be maintained by heterochromatin formation and the sub nuclear positioning of *var* genes is important for activation [252] [257] [258] [259]. By DNA-FISH (fluorescent in situ hybridization) Howitt et al showed that an active episomal *rifin* promoter colocalizes with an active *var* promoter suggesting that these gene families may use the same sub nuclear expression site. Antisense long noncoding RNAs have also been suggested to regulate *var* gene activation [260] but this has not been show in the regulation of *rif* genes. Overall, there is little knowledge on how exactly the expression of *rif* genes are regulated.

1.9.4 STEVOR and other variant surface antigens

The sub -telomeric variable open reading frame (*stevor)* is similar to *rif* genes as they consist of two exon encoding small proteins, around 30-50 kDa, similar in size to the RIFIN proteins. The *stevor* gene family is smaller than the *rif* family and is comprised of 30-40 copies per genome [191]. Exon 1 encodes a start codon and a putative signal peptide, exon 2 encodes the remainder of the protein including potential trans membrane domains and an extracellular highly polymorphic domain [261]. Stevor and rif genes are suggested to be a part of a larger superfamily, Plasmodium interspersed repeats (pir) which in *Plasmodium vivax* are referred to as vir, in *Plasmodium knowlesi* as kir and the *cir/bir/yir* family in three rodent malaria species [241]. STEVORs are detected at multiple stages of the parasite life cycle suggesting that they mediate multiple distinct functions [262]. Similar to RIFINs they are trafficked to the infected erythrocyte membrane [263] and antibodies are naturally acquired to both proteins [248] [264]. Gene expression peaks at 22 to 28 hours post invasion and proteins are present in the trophozoite to schizont stage as well in gametocytes and sporozoites [265] [266] [267]. They have also been detected in the apical end of merozoites [263] [234] [268]. Their function is not fully known, experiments by quantitative PCR and STEVOR specific Ab assay suggest that only a subset are expressed in each parasite and that the expression is clonal variant [267] [269]. They are supposedly trafficked to the infected erythrocyte membrane [115] [263] and recent published data show that some of the STEVOR proteins are exposed at the erythrocyte membrane similar to

RIFINs and that they point to the lumen away from the cytosol in MCs [227]. Another publication also suggests the involvement of STEVOR in mediating rosetting independently of PfEMP1 through the interaction with Glycophorin C (GPC) on the RBC surface. This interaction also provided a relative growth advantage by protecting the merozoites from invasion-blocking antibodies [160]. The expression of STEVOR proteins has also been correlated to increase the increase in membrane rigidity [270].

1.9.4.1 SURFIN

The surface associated interspersed (*surfin)* gene family consists of 10-12 genes (including a few pseudogenes) located close to the sub-telomeric regions. Specific SURFIN proteins have been detected in Maurer's clefts, exported to the red blood cell surface and on the apical end of the merozoites where they have been suggested to be responsible for the reversible association with target erythrocytes [271] [272].

1.9.5 The transcriptional landscape of the intra-erythrocytic cycle of P. falciparum

Gene expression profiling has been key in providing the molecular understanding of the intra developmental cycle (IDC) of *P.falciparum*. Bozdech et al. provided a 1hr time-scale resolution of *P.falciparum* HB3 strain using microarray technology and it is the first comprehensive investigation of the IDC [273]. The study established that at least 60% of the genome is transcribed and it shows a highly periodic expression of genes throughout the IDC with most of the genes having a peak in transcription at the timepoint for their specific function during the IDC. This pattern of transcription was reaffirmed by other studies using RNA sequencing as well [274] [275] and was also found to be conserved in geographically diverse isolates [276]. This conserved transcription of genes important for the specific basic metabolic processes has therefore been called a "hard wired" transcription with an incapacity for changing RNA levels in response to specific metabolic perturbations [277]. Although it is clear that sexual commitment and drug-induced quiescence are examples of individual parasites responding to extracellular signals thus changing their transcriptional activity.

The differences in gene expression that has been observed between strains is largely due to genes expressed on the *P.falciparum* cell surface involved in cell adhesion, defense and immunity, antigenic variation and host-pathogen interaction [273] [274] [201]. Although,

Rovira Graells et al., also observed that parasite populations derived from a single parasite (clonal) had a variable transcription of genes involved in metabolic processes such as lipid metabolism, nutrient import, protein folding or in erythrocyte invasion. This clonal variant expression give rise not only to antigenic variation but also to functional variation that may increase the parasite population´s potential to adapt to different environmental stress [278] [276]. The authors suggest that the parasite utilize a stochastic gene expression so called bet-hedging strategy [279] for a large number of genes to modulate fitness and mediate adaptation to changes in the environment e.g. temperature related to febrile episodes or the genetic background of erythrocyte receptors in the host [276].

2 SCOPE OF THE THESIS

The general aim of this thesis was to study the rosetting phenomena and in more detail investigate the topology and function of the RIFIN proteins.

SPECIFIC AIMS

The specific aims of each paper included in this thesis are to:

i. Determine the topology of A-RIFIN and B-RIFIN and their role in rosetting (Paper I)
ii. Study the functional role of rosetting as an immune evasion strategy (Paper II)
iii. Utilize a novel single-cell RNA sequencing tool to explore its utility for malaria research. Study transcription of virulence antigens involved in i) rosetting and ii) in sexual commitment (Paper III)

3 EXPERIMENTAL CONSIDERATIONS

All material and methods used for the experiments presented in this thesis are described in Papers I-III. Methods, important to understand the general part of the thesis are described in this section.

3.1 PARASITES AND *IN VIVO* CULTURE CONDITIONS

All isolates and strains were cultivated using standard methods as developed by Trager and Jensen (Moll et al., Trager and Jensen 1976) with the modification that they were cultivated in a gas mixture of 90 % NO_2, 5% O_2, and 5% CO_2 and shaking incubation replaced the static candle jar technique. This improved method has been shown to reduce the number of multiple-infected red blood cells. Patient isolates were collected in Kampala, Uganda [26]; establishment of cultures was carried out as described in Ribacke et al., 2013 [280]. All parasite cultures were kept under shaking conditions during all experiments.

The rosetting phenotype is maintained by enrichment over a Ficoll-gradient (Methods in Malaria Research. 6th Ed: Moll, Kaneko, Scherf & Wahlgren). In Paper III the transgenic 3D7 P2G12 tDT-164 parasite strain is used. It expresses the tDT-164 red fluorescent reporter gene under the promoter of the early sexual commitment gene ETRAMP10.3 (PF3D7_1016900). This clone has a preference of rosetting in blood group A compared to blood group O (rosetting rate in bg A 50-75% and in bg O < 5 %). Parasites were kept synchronous by using 5 % sorbitol (w/v) to synchronize early rings and additional MACS sorting of the late trophozoite/schizont stages (Methods in Malaria Research. 6th Ed: Moll, Kaneko, Scherf & Wahlgren).

Rosette size was determined by counting the number of bound erythrocytes in 100 rosettes by staining the parasite culture with Acridine Orange and microscopic investigation. Similar, multiple infections were investigated by staining the parasite culture with Acridine Orange and microscopic investigation of 300 iRBC.

3.2 EXPRESSING RIFIN IN CHO CELLS USING THE PDISPLAY VECTOR

The pDisplay vector from Invitrogen targets the protein of interest to the extracellular surface of the cell anchored with one vector specific TMD Genes encoding A-RIFINs PF3D7_0100400, PF3D7_1101100, and B-RIFIN PF3D7_0900500 were PCR amplified from amino acid 38 to 329 and cloned into BglII and SalI sites in a pDisplay vector (Invitrogen, USA). Proteins expressed from the pDisplay vector were fused at the N

terminus to the mouse Ig κ-chain leader sequence, to direct the protein to the secretory pathway, and at the C terminus to the platelet-derived growth factor receptor (PDGFR) trans membrane domain, which anchors the protein to the plasma membrane, displaying it on the extracellular side. The expression construct held sequences encoding a hemagglutinin A tag (HA) at the N terminus and a MYC tag at the C terminus of the RIFINs. All the constructs were confirmed by sequencing using Big Dye Terminator v3.1 and ABI Prism 3130xL Genomic Analyzer capillary sequencer (Applied Biosystems, California, USA). The pDisplay constructs containing genes encoding A-RIFINs PF3D7_0100400, PF3D7_1101100, or B-RIFIN PF3D7_0900500 were transfected into CHO cells using Fugene (Roche biosciences, Switzerland) according to the manufacturer's protocol.

3.3 ERYTHROCYTE BINDING ASSAY

Transfected or control cells were seeded in 6- or 12-well culture plates overnight and grown until they were 40–50% confluent. Aliquots of blood group A or O RBC were washed twice in PBS and added to the cells at 2% hematocrit in complete growth media. The cells were incubated with erythrocytes at 37 °C in a CO2 incubator for either 5 or 15 h and subsequently washed extensively three times with RPMI to remove unbound RBCs. Binding was assessed by microscopy (Diaphot 300, Nikon, Japan). The binding was calculated as the percentage of CHO cells that bound RBC. Further, binding was also estimated as to the number of RBC bound (3–4 or >5) per 1,000 cells using a Nikon Diaphot 300 (Japan) microscope at a magnification of 100×. 3-α-N-acetylgalactosaminidase (Azyme) and 3-α-galactosidase (Bzyme) were used for the conversion of blood group A and B, respectively, to blood group O

3.4 GENERATION OF IMMUNE SERA

E. coli–purified A-RIFIN (PF3D7_0100400) was used to repeatedly immunize rats, rabbits and a goat using Freund´s incomplete adjuvants (Agrisera, Vännäs, Sweden). The C-terminal region of the RIFIN (RYRRKMKLKKKLQYIKLLEE) coupled to KLH was used to immunize rabbits using Freund's incomplete adjuvant. The sera were collected after the fourth immunization and the IgGs were purified and used in the various assay.

3.5 BLOOD GROUP PREFERENCE IN THE FORMATION OF ROSETTES

Infected RBC (in blood group O) older than 24h post infection. were enriched by MACS as described earlier and re suspended in malaria complete medium with 10% human serum (MCM S+). Unparasitized RBC of blood group A and O were each labelled with PKH of one out of two different colours (Sigma, MINI67-1KT, MINI26-1KT). Staining was carried out using PKH67 for green and PKH26 for red labelling. 1 µl of RBC was washed twice with RPMI and mixed with 125 µl Dilutent C, thereafter a mixture of 125 µl Dilutent C plus 0.5 µl PKH 67 or 26 was added and incubated for 5 min at RT; 250 µl of PBS/FCS 3% were added and the cells washed twice with RPMI. RBC were re suspended in (MCM S+) to 5% hematocrit; RBC of both blood groups labelled in two different colours were mixed to same percentage and purified iRBC were added to a final parasitemia of 10%; formation of agglutinates of MACS enriched iRBC was mechanically broken by passage through a needle directly before addition to the experiment. Formation of rosettes was allowed for 60 min at RT and the percentage of blood group A or O RBC was determined by microscopic investigation of 100 individual rosettes.

3.6 SINGLE CELL RNA SEQ

Single-cell RNA sequencing (sc-RNA-Seq) represents a milestone of technological advancement in the transcriptomics field. The new method has had tremendous impact on various disciplines of biology and clinical research including developmental biology [281] cancer [282] stem cell research and transcriptional kinetics [283].

In this work, samples were collected and processed at four separate occasions. The sampling was done at 10, 14, 20, 24, 32, 38, 46 ± 2 hours post invasion (p.i). At each time point 200 ul of the parasite culture was removed and washed 2 x in 37 °C pre-warmed PBS. In order to easily identify iRBC we stained the cultured cells with MitoTracker Green fluourescent dye (Life technologies). From the washed culture 10 µl of packed RBC was stained with 0.5µl MitoTracker, in 1 ml warm PBS for 15 min at 37 C. Then washed 3x in warm PBS and re-suspended in 0.5 ml PBS. To compare the impact of the fluorescent dye a number of samples were stained with the DNA-binding dye Vybrant® DyeCycle™ Green (Life technologies). Parasite culture was washed as previously described and 10 µl of packed RBCs were stained with 1µl Vybrant in 1 ml PBS for 15 min at RT.

For collecting single infected RBCs, 1µl of the stained parasite cultures were diluted in 2

ml PBS in a petri dish. Since erythrocytes are fragile cells, we picked individual infected RBC using the Cell Sorter. Thus we were able to minimize physical stress, time span of the collection process and monitored the picking process real time the to ensure to obtain individual, single infected RBC´s only. The isolated single cells were collected in 200 µl to PCR tubes containing 2.5 ul Smart-seq2 lysis buffer. Replicates of bulk samples were prepared by taking 1 µl of RBC suspension to 2.5µl lysis buffer. The samples were spun down and frozen directly at -80C for efficient lysis. *P.falciparum* full length cDNA was obtained following the improved Smart-seq2 protocol [284]. The samples were processed according to Smart-seq2 protocol. Full-length cDNA from single iRBC was amplified using 23 PCR cycles and bulk samples were amplified using 19 amplification cycles. In order to generate Illumina compatible sequencing libraries, full-length cDNA´s were tagmented by in house Tn5 transposase following the protocol of Picelli et al. [285]. All libraries were sequenced on Illumina HiSeq 2000.

4 RESULTS AND DISCUSSION

4.1 PAPER I: RIFINS ARE ADHESINS IMPLICATED IN SEVERE PLASMODIUM FALCIPARUM MALARIA

In Paper I we show that the *P.falciparum* encoded repetitive interspersed families of polypeptides (RIFINs) are expressed on the surface of infected red blood cells (IE). They show a higher affinity for RBC of blood group A than blood group O RBC and they form larger rosettes with group A than group O RBC. Further the RIFINs are likely to be involved in microvascularute binding of IE.

Earlier studies demonstrated that, RIFINs are indeed expressed on the surface of infected RBCs in mature parasites [225] [226] [231] and serum antibody titers from semi-immune adults were found positive to react with recombinant RIFIN proteins. The increased presence of anti-RIFIN antibodies in asymptomatic children compared to severe malaria cases [248] [249] as well as the up regulation of RIFINs in cerebral malaria [245] clearly suggest they play an important part in the severe progression of the disease. In this work we define the RIFIN protein topology, the expression of RIFINs on the surface of infected RBCs (iRBCs) and their involvement in the rosetting phenomena, a major pathogenic mechanism underlying the severe forms of malaria.

Due to their small size and hydrophobic domains it was important to first determine their topology and the number of trans membrane domains in order to understand what part of the protein is presented to the extracellular environment and therefore possibly involved in adhesion. Previously most of the RIFINs were described as two trans membrane domain (TMD) proteins by sequence analysis of hydrophobic regions but this had not been tested with molecular methods. In order to experimentally define the TMDs of RIFIN we tested an *in vitro* protein expression system using dog pancreas microsomes (DPRM). This is an artificial system that uses N-linked glycosylation states to assess the integration of membrane proteins into the equivalent of an endoplasmic reticulum membrane. The DPRM contain N-linked glycosylation machinery that will modify acceptor sites within segments of the protein that translate to the lumen of the DPRM. Two RIFINs from the parasite strain 3D7 annotated genome RIFIN-A (PF3D7_0100400) and RIFIN-B (PF3D7_0900500), were modified by removing or adding glycosylation sites and *in vitro* expressed in the DPRM system. The parts of the proteins, which are translated into the lumen, can then be mapped

by the glycosylation pattern and the number of TMDs can be determined. Expression results of both, the RIFIN A and B in the DPRM system, showed the presence of only one trans membrane domain in both proteins.
To further investigate if RIFINs bind to uninfected red blood cells and mediate rosetting we then expressed the same A and B RIFINs and an additional A RIFIN (PF3D7 _1101100) on the surface of Chinese hamster ovary (CHO) cells using the pDisplay vector (Invitrogen).

To test whether RIFINs cells bind RBCs we performed binding assays with RBC of different phenotypes on the CHO expressing RIFIN. Previous experiments showed that enzymatic removal of PfEMP1 did not reduce rosetting in blood group A, indicating that there is another ligand involved in blood group A binding. Therefore, we tested the possibility of RIFINs binding to blood group A and O RBCs. We found that the CHO cells expressing the A-RIFIN bound large numbers of group A RBCs (up to ~25 RBCs per CHO cell) whereas the binding of group O RBCs was less pronounced, comparable to CHO cells expressing the N-terminal domain of PfEMP1 (DBL1α; IT4var60) known binder of RBC and a positive control in this study. No specific binding was observed to CHO cells expressing B-RIFIN or CHO cells expressing a different PfEMP1 (DBL1α of PfEMP1-FCR3S1.2var1), which does not bind to RBCs and therefore used as negative control. The lack of binding of the B-RIFIN is in agreement with earlier studies, which show that they are localized inside the parasite–cytosol [230] [231] and therefore can be suggested not to be involved in RBC binding. To further confirm that the A-RIFIN binding was due to the blood group A antigen, we performed binding assays with enzyme treated RBCs where the terminal α3 *N*-acetylgalactosamine (GalNAc) residue of the group A oligosaccharides was removed. This converts the characteristics of A RBCs into O RBCs. The CHO cells expressing A RIFIN bound the converted A RBCs in a similar level as normal O RBCs indicating that the A antigen is important for the binding.

We subsequently tested two different naturally occurring sub types of blood group A defined by the level of A-antigen expression. Group A_1 RBCs express approximately five times more A antigen on the surface of the RBC than A_2 RBCs. The amount of RBCs binding to A-RIFIN CHO cells correlated with the level of group A antigen expression binding since significantly more group A_1 than group A_2 RBCs were found bound. Similarly, when we studied the enzyme treated RBCs in the P.falciparum clone FCR3S1.2 they formed smaller rosettes comparable to those formed by group O RBCs.

The dominant PfEMP1 expressed in this clone is the IT4var60 variant identified in previous experiments by Albrecht et al [156], also studied in paper II. To identify the RIFINs

expressed in the FCR3S1.2 clone we performed a RNA-Seq analysis at 10 h intervals throughout the intra erythrocytic cycle starting from merozoite invasion. The results show high expression of a single *rif* gene (PFIT_bin05750) and low expression of 84 *rif* genes. The maximum expression was detected at 20h post invasion.

The RNA-Seq data supports the assumption that there is one dominant *rif* gene expressed implicating that *rif* genes may be mutually exclusive expressed comparable to *var* gene expression dynamics [220]. Further, to confirm the binding of A-RIFIN to RBCs on protein level we expressed the full-length extracellular recombinant A-RIFIN PF3D7_ 0100400. After protein purification we found similar protein species identified by SDS-PAGE, immunoblotting or LC-MS/MS. A pattern detected in early experiments in *P.falciparum* [225]. This data together with the RNA-Seq data supports the assumption that there is one dominant *rif* gene expressed and post translationally modified, implicating that *rif* genes much like *var* genes, are mutually exclusive expressed.

The expressed RIFIN protein bound to both A and O RBCs with higher avidity to A compared to O in compliance with the experiments in CHO cells. We also generated antibodies to the full length A-RIFIN of the 3D7 parasite and these cross-reacted with the heterologous RIFIN expressed in FCR3S1.2 parasite in both immunoblotting and immunofluorescence analysis. We detected RIFINs at the parasitophorous vacuole, Maurer's clefts and to the membrane of iRBCs and live, unfixed iRBCs. The pattern of the staining was similar to that obtained with antibodies to PfEMP1 (ITvar60) although when we co stained using both antibodies they were only partially overlapping. This indicates that PfEMP1 and RIFINs are exported and displayed on the surface of the iRBC in different manners. The RNA-Seq data also showed that their time of maximum transcription differs. A crucial finding was that the RIFIN-specific IgG disrupted rosettes of blood group A but not O in the FCR3S1.2 parasites. This provides further evidence that the A RIFIN expressed in this parasite is the major factor for binding blood group A.

The ABO antigens are expressed not only on RBCs but also on endothelial cells. To further study the pathophysiological role of A-RIFIN in microvasculature sequestration we first transfected two different strains of *Plasmodium* parasites selected for low rosetting (FCR3S1.2-low and R29-low) with the FCR3S1.2 *rif* gene PFIT_bin05750 tagged with a GFP reporter gene. Both transfectants increased their rosetting rate approximately 40 % compared to the non-transfected control. The R29 transfected parasites displayed a higher rosetting rate in blood group A compared to O while as was the case with the FCR3S1.2

parasites transfected with the RIFIN. The FCR3S1.2-RIFIN-GFP parasites were cultured in blood group A human RBCs and then injected into Sprague_Dawley rats. The results showed significantly more binding of the parasites expressing RIFIN in the microvasculature than the control RBCs.

To investigate the receptor involved in the blood group O binding we treated O RBCs with different enzymes cleaving various receptors on the RBC surface and studied the binding in parasites and CHO transfectants. These experiments showed that RIFIN binding was mediated by sialic acid on glycophorin A (GPA).

Binding experiments with human RBCs naturally deficient of glycophorins A, B or C (GPA, GPB, GBC) also showed that En(a-) RBCs deficient for GPA bound much less compared to the other blood types. Interestingly, En(a-) expressing the blood group A antigen bound significantly more supporting once again that the A antigen is the major receptor for A-RIFIN.

Overall, this study provides the first evidence that A-RIFINs expressed on the surface of the iRBC play an important part in rosetting with specific preference for the blood group A antigen. Together the data indicates that RIFINs have a fundamental role in the development of severe malaria.

4.2 PAPER II: EVASION OF IMMUNITY TO PLASMODIUM FALCIPARUM: ROSETTES OF BLOOD GROUP A IMPAIR RECOGNITION OF PFEMP1

The Plasmodium parasite has developed several ways to evade the host immune system during the erythrocytic life cycle. By expressing variant surface antigens, it can bind to endothelial cells and sequester in the microvasculature escaping clearance by the spleen. In this work, we explore the hypothesis that the rosetting phenomena protect the infected RBC by hiding surface molecules that could be targeted by the immune system.

We analyzed a panel of parasite isolates and clones and their capacity to form rosettes in different ABO blood groups. These included four laboratory parasite clones (FCR3S1.2, R29, PaVarO, 3D7S8.4) and four patients isolates (UKS41, UKS55, UKS86, UKM104) all having a rosetting phenotype. Rosetting is mediated by both a subgroup of PfEMP1 adhesins, where the RBC binding is assigned to the N-terminal DBL1α_1 domain [167] and

by RIFINs of subgroup A (Paper I). Each parasite strain expresses different variants of these surface antigens. The laboratory clone FCR3S1.2 expresses the PfEMP1 variant ITvar60 that mediates rosetting of RBC of all ABO-blood groups since it binds to heparan sulphate present on all RBC [156] while the RIFIN variant IT-5750 primarily-mediates blood group A rosetting but also binds to sialic to mediate minor binding of all RBC (Paper I). The PaVarO clone has similarly a marked preference for binding to group A over O and expresses the PfEMP1 variant VarO [167]. The R29 and 3D7S8.4 clones however both express var genes of group A NTS-DBL1a PfEMP1 domain that are not defined as either A or O preferring. The RIFINs that are expressed in the PaVarO, R29 and 3D7S8.4 clones, if any, are not known yet.

First, we assayed the formation and the size of rosettes of the different parasite clones in blood group A and O in parallel. The laboratory clones FCR3S1.2, PaVarO and the isolates UKS41 and UKS55 formed significantly bigger rosettes in blood group A as compared to O.

We then assayed the preference of different parasites for the A and O blood group by incubating iRBC with A and O red blood cells pre labelled with different dyes. All of the investigated parasites showed a preference for binding to blood group A RBCs. Although, the tendency of the R29 clone was much less compared to the other parasite clones. We then assayed the sensitivity of the rosettes in the FCR3S1.2, R29, PaVarO parasites to strain specific PfEMP1-DBL1α monoclonal antibodies (mAb) and polyclonal antibodies at two concentrations (100 and 50 ug/ml). Patient isolates could not be included due to the lack of strain-specific PfEMP1-DBL1α antibodies. In FCR3S1.2 and PaVarO the blood group O rosettes were disrupted while the rosettes of blood group A were not. In contrast rosettes of the R29 clone showed equal sensibility to the antibodies in both blood groups. Similar results were obtained with the polyclonal antibodies and similar levels of PfEMP1 molecules were detected on the iRBC surface using FACS independent of the blood group that the parasites were cultured in. This indicates that the formation of rosettes in blood group A impairs the binding of antibodies targeting the PfEMP1 molecule on the surface of the iRBCs. This, also suggest that other antigens such as RIFIN contribute to group A rosetting and protect the iRBC from antibodies. *In vivo* the rosetting may not make it more difficult for the immune system to develop antibodies against surface exposed antigens but to access them.

However, when strain specific antibodies targeting PfEMP1 are present during the

maturation of parasites from ring stage to trophozoite stage the formation of rosettes are inhibited in both blood groups. This is possibly due to the large size of PfEMP1 (280,000 kDa). When antibodies are bound to the PfEMP1 molecule they can sterically hinder binding of other antigens such as the small RIFIN (40,000 kDa) to RBCs. It also shows the complexity of rosetting, specifically to A RBC where several antigens and receptors are involved suggesting a broader composition of protective antibodies needed to inhibit these interactions.

In these *in vitro* cultures, we did not detect any advantage of rosetting for the parasites such as growth, invasion, multiplication-rates or multiple infected iRBCs. However, if this is also the case *in vivo* cannot be determined yet. Additionally, non immune IgM bound to the surface of FCR3S1.2 iRBCs has an important role in rosetting [221, 286], therefore we tested if there was any difference in the binding of IgM to iRBC in blood group A and O. But no difference was detected.

In summary we show that rosettes of blood group A protect the parasite from antibodies targeting the PfEMP1 molecule at the surface of the iRBC. Giving a possible explanation why the ABO blood group antigens are so important for the outcome of a malaria infection and how rosetting may contribute to this.

4.3 PAPER III: TRANSCRIPTIONAL ANALYSIS OF PLASMODIUM FALCIPARUM ERYTHROCYTIC DEVELOPMENT AT CELLULAR RESOLUTION

The recent advances in single cell RNA-Seq technologies have so far been unexplored in the field of infectious diseases such as malaria. Therefore, in this work we investigated the utility of RNA-sequencing of single *P. falciparum* infected red blood cells. We determined genome-wide expressional profiles of *P. falciparum* infected red blood cells (iRBCs) and population samples respectively, sampled at six different time points during the 48 hour IDC cycle. We have validated the dataset, characterized the transcriptional profiles and identified sub-states during IDC in a synchronized lab-adapted parasite line (3D7). For all single-cell and population samples, we generated sequencing libraries applying the Smart-

seq2 protocol [284] followed by an in house developed Tn5 tagmentation protocol for Next Generation Sequencing on the Illumina platform [285].

We computed expression levels for malaria genes in RPKMs (Reads Per Kilo base per Million mapped reads) using uniquely mapping reads only. The number of detected genes varied with time point. The relatively lower number of detected genes in the early stage samples (R and LR) can be attributed to several factors. The ring stage is the most transcriptionally inactive and therefore the most challenging to profile. However, the low number of genes expressed in the early trophozoite category was unexpected. This pattern may reflect a sub-state of lower gene expression since this pattern was observed in all the independent experiments. The correlation between samples increased with the progression through IDC (post-ring samples median correlation coefficient >=0.2). Which could reflect an enhanced transcriptional state with up-regulation of cellular functions towards a more defined transcriptional profile.

To validate if the single cell libraries contained a biological signal we analyzed the expression of genes that are dynamically regulated during *P. falciparum* development. The single cell libraries showed the expected temporal expression dynamics of known marker genes at the different IDC time points and in general, single cell transcriptomic profiles correlated with their corresponding population samples.

The heterogeneity observed among individual parasites is likely due to the limits and challenges of synchronizing large parasites on bulk level and biological variation in developmental timing of individual infected cells. We overcome this obstacle by unsupervised hierarchical clustering of the single cells and recapitulate their true *in vitro* IDC stage.

In summary we show that biological information from RNA-sequencing of single-cell *P. falciparum* infected red blood cells can be obtained and the developmental stage of the individual iRBCs can be determined.

5 CONCLUDING REMARKS

Malaria is still a major health burden. The dawn and spread of resistant parasites to available treatments is a major threat to reaching the goal of eliminating malaria.
In order to develop effective vaccines and new treatments to decrease the disease mortality and to reach this goal we need to increase our understanding of the biology of the *P.falciparum* parasite. To do this we also need to develop new methods for malaria research. The work presented in this thesis give some new clues of parasite biology and a method that provides the ability to study the transcriptome of individual parasites.

In Paper I we present the first functional description of the RIFIN protein family. We show that A RIFINs are expressed on the surface of the infected RBC in the trophozite stage of the erythrocytic cycle, where they mediate rosetting. We also show that parasites highly expressing RIFINs mediate microvasculature sequestration. These are major pathological features of severe malaria that has had an enormous impact on the human population and most likely the global distribution of the ABO blood groups. However, when one question is answered it gives rise to several more. The RIFIN family is the largest multi gene family in *P.falciparum* with about 200 copies per haploid genome. In our work we have studied three *rif* genes and one in more detail The question is do all A RIFIN bind to blood group A specifically or do they, as the different PfEMP1 proteins have specific binding characteristics? Expression of the RIFINs has also been detected in the other stages of the parasite life cycle, as in the merozoite and gametocyte-what are their functions there? Another important question that needs to be further studied is to understand how the *rif* genes are regulated. These are just a few questions that arise and overall there are many directions to take to further explore the RIFIN family of proteins.

In Paper II we show that parasites in blood group A inhibit antibodies targeting the PfEMP1 molecule on the surface of the infected RBC suggesting that the large and tight rosettes formed in blood group A not only sequester the parasites in the microvasculature due to size, but also acts as an immune-protective shield. Hiding parasites from antibodies and phagocytic cells that otherwise would lead to clearance by the spleen. It also provides a more complex picture of the parasitic surface antigens involved in rosetting and how the ABO blood group contributes to this. To increase the understanding of this complexity and

identification of various expressed surface antigens, such as the RIFINs in different rosetting parasite clones and production of strain specific antibodies to these is needed.

In Paper III we confirm the utility of RNA-Seq of single infected RBCs and explore the transcriptional profile of individual parasites during the intra erythrocytic cycle. We show that in spite of the minute mRNA content the biological information is retained in individual parasites. This opens an opportunity to study various aspects of the biology at single cell level. Exploration of important features such as, mutual exclusive expression of surface antigens, for example the RIFINs, drug induced quiescence and the transcriptomic profile of sexually committed parasites or parasites at different stages of gametocytogenesis.

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